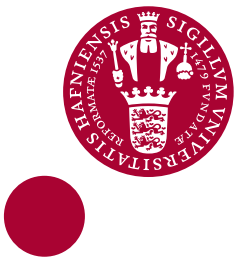

Mycoplasma bovis pathogenesis, diagnostic methods and epidemiology of relevance for control and prevention

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Publication date:
2016

Document version
Peer reviewed version

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Summary

Mycoplasma bovis introduction to and spread within cattle herds is difficult to predict, and no vaccines provide efficient prevention today despite the fact that this infection severely affects animal welfare and leads to economic losses in cattle farms worldwide.

The most common transmission routes are direct cattle-to-cattle contact, contaminated milk and milking equipment, and aerosols moving between animals over short distances (few meters), so any prevention method that can mitigate those routes will reduce the size and duration of outbreaks. Less common transmission routes include semen from infected bulls and perhaps long-distance airborne pathogens being spread between outbreaks and susceptible farms.

Prevention and control methods have to focus on management and biosecurity measures that maximise resilience of the farm system and the animals' resistance against the disease in case the herd becomes exposed to *M. bovis* - or new strains of *M. bovis*. Luckily this also has great benefits on the general health and production in cattle farms. The general prevention measures can to some extent be supported by diagnostic testing prior to movement of cattle, even though it is not possible to point to an accurate testing procedure that can entirely rule of infection in animals or herds. Hence, trade and close contacts always pose a risk and measures to reduce the risk including the consequences upon introduction should be used.

Essential elements in systematic disease control and prevention

Successful systematic control and elimination of infectious diseases in livestock populations rely on a number of prerequisite elements that are common for all infectious diseases. Yet, some elements are more complicated to address or less well investigated for *M. bovis* compared to other diseases that have been successfully eradicated from cattle populations in the Nordic countries. Therefore this disease frustrates many farmers and their local veterinary advisors. This presentation provides an overview of important elements of *M. bovis* relevant for control options. It also provides some suggestions for prevention of *M. bovis*-related disease in cattle herds, where little or no evidence is available in the current literature.

The pre-requisite elements that are common to all diseases have been described by Houe et al. (2014):
1) Motivation must be present for stakeholders to get engaged (e.g. animal welfare, economics and human risk related to zoonotic infections);
2) Necessary biosecurity measures must be understood and well-established to stop or at least mitigate intermittent or continuous transmission of infection. This also implies a solid understanding of the agent characteristic and pathogenesis of the disease;
3) Purpose specific and systematic test-strategies are usually needed to be able to identify infectious animals for culling or risk-mitigating management, and to classify herds according to disease or infection status or for documentation of freedom from infection;
4) One or more pilot studies should have been carried out to provide proof of concept that identified biosecurity measures and test-strategies are feasible and efficient in a practical setting;

5) Resources such as laboratory capacity, suitable databases and sufficient will to comply with recommendations among essential stakeholders, plus means to support the program by regulative or legislative means must be (made) available;
6) The decision on whether to aim for control or eradication is very complex, so visionary and persistent leadership is essential. All the needed information about the disease and the context must be evaluated before a systematic programme is initiated.
7) Communication must ensure that all participants and stakeholders continuously have relevant and updated information. For example, farmers must have operational guidelines for biosecurity measures and veterinary practitioners must have exact knowledge of sampling animals for laboratory testing;
8) Follow-up investigations must accompany the programme once it is running. For instance, re-infection of cleared herds may reveal unknown ways of transmission, and it must also be considered if new legislation is necessary to support the programme as there always seem to be certain herds that are particular difficult to handle.

After successful elimination of an infection from a farm or national eradication of an infection, it should be considered exotic and the focus change to 'preparedness in peacetime' including occurrence in neighbouring farms and countries and methods for surveillance and early detection of infection (Houe et al., 2014). Below these elements are addressed for *M. bovis*.

Agent characteristics

Mycoplasmas belong to the bacterial class Mollicutes, the smallest prokaryotic cells capable of replicating themselves. *M. bovis* is host-adapted to cattle, where it can be anything from a simple commensal to a pathogen opportunistic or primary pathogen (Maunsell et al., 2011). The only other species in which clinical signs have been experimentally produced is sheep (Bocklisch et al., 1991). However, *M. bovis* has also been isolated from a naturally infected goat with mastitis (Ayling et al., 2004) and pigs after spill-over of pathogens during an outbreak in Austrian Alp pastures (Spergser et al., 2013).

Antimicrobial susceptibility

Mycoplasmas have no cell wall and are therefore resistant to antibiotics that interfere with cell wall synthesis such as penicillins. They are also naturally resistant to polymyxins, sulfonamides, trimethoprim, nalidixic acid, and rifampin. In addition, field studies often report strains resistant to other types of antimicrobials including tetracyclines. Macrolides that are mycoplasmastatic provide the host immune response with an opportunity to combat the infection. However, *M. bovis* has defence mechanisms including an ability to vary its surface proteins and form biofilms. The types of antimicrobial resistance patterns vary regionally, but overall the best treatment effects in cattle are currently obtained using macrolides, lincosamides, and pleuromutilins (Lysnyansky and Ayling, 2016). However, early onset of treatment is crucial for it to have any effect. Thus, proper daily surveillance of all cattle in the farm is essential. During an outbreak, extra surveillance procedures may have to be put in place, such as checking the general condition, temperature and feed uptake of individual animals to be able to detect diseased animals to isolate and treat early.

Survival in semen and embryos

M. bovis can remain infective for years in deep-frozen bovine semen for artificial insemination, and antimicrobial treatment of the semen may not always remove the agent (Visser et al., 1999). This was also recently seen, when *M. bovis* was introduced to Finnish farms with semen from an infected bull. Even though artificial insemination with *M. bovis*-contaminated semen can be a source of infection of the female bovine genital tract, it is uncommon in countries with strict physical and antimicrobial treatment of semen used for commercial purposes (Garcia et al., 1986; Petit et al., 2008), and OIE provide elaborate guidelines to minimize the risk.

An experimental in-vitro study found that *M. bovis* microinjected into embryos remained infectious even after the embryos were thoroughly washed and treated with trypsin and combinations of penicillin, streptomycin, lincomycin and spectinomycin, or gentamicin, tylosin, lincomycin, and spectinomycin (Bielanski et al., 1989). However, the presences of *M. bovis* in embryos do not necessarily lead to reproductive or disease problems.

Because treatment does not provide a 100% safe way to mitigate the risk of transmission by artificial insemination, it is important that the presence of any clinical signs that might be caused *M. bovis* in the herd must be suspected as *M. bovis*, and animals exhibiting such clinical signs - even if only mild signs - should not be used for collection of semen or embryo transfer until several weeks after clinical signs have ceased in the herd. PCR-methods to test semen before use exist, but is an extra expense in the breeding programmes (McDonald, 2012; Naikare et al., 2015).

Environmental survival and reservoirs

One study has reported fairly long survival times outside the host under cold conditions in investigations conducted with artificially contaminated materials: *M. bovis* survived on sponges for 57 days, in milk for 54, on straw for 20, and on wood and in water for 17 days at 4°C, whereas the survival period on these materials dropped to 1-2 weeks at 20°C, and to 1 week at 37°C (Pfützner, 1984).

In a 6 week study of the potential infectiveness of naturally contaminated sand from a dairy herd no evidence of transmission from the contaminated sand (200 and 32,000 cfu/g) was found in the 166 samples collected from the upper airways and ears of the 12 study calves, and no signs of pathology was present at necropsy at the end of the study (Wilson et al., 2011). Another study suggested that recycled bedding sand from a herd with a clinical *M. bovis* outbreak might pose a risk of udder infections unless the sand was treated (Justice-Allen et al., 2010).

Airborne transmission

Well-founded studies of airborne transmission of *M. bovis* seem to be lacking, so it is necessary to look at literature about other mycoplasma species to try to derive a best guess of the airborne transmission potential of *M. bovis*. Survival of *Mycoplasma pneumonia* in air particles was found to be best at very low and at very high humidity levels. In contrast, at 60 and 80% relative humidity levels less than 1% of the organisms survived over a 4-hour observation period (Wright et al., 1969; Wright et al., 1968). In general, increasing temperature was found to decrease survival time. The strong effect of humidity provides a good explanation for why barn ventilation plays an important role in the prevention of *M. bovis*-associated disease in cattle farms.

In an experimental on long-distance airborne transmission of *Mycoplasma hyopneumonia*, 5% of the air samples collected between 3 and 9 km away from a highly infectious farm was found positive with infectious pathogens indicating that most pathogens do not survive, but during outbreaks of disease long-distance spread is feasible (Otake et al., 2010). However, the air exhaust from pig farms might contain quite different air compositions and concentrations of bacteria than most cattle farms, which are often more open and naturally ventilated, so the results from studies of mycoplasma transmission in pigs may not extrapolate well to cattle conditions.

It is important to remember that the risk of airborne transmission between farms depends on many other factors than temperature and relative humidity, e.g. dust density, ventilation types, wind conditions and structural or geographic obstructions between farm premises (Callan and Garry, 2002).

Proper ventilation serves eight primary functions:

1) decreases the airborne pathogen concentration
2) eliminates noxious gases (ammonia, hydrogen sulfide, carbon dioxide, carbon monoxide, methane)
3) decreases airborne dust contamination
4) decreases airborne endotoxin levels
5) maintains optimum ambient temperature
6) maintains optimum environmental humidity levels
7) eliminates drafts
8) eliminates areas of stagnant air

It should also be kept in mind that as the airborne pathogen load rises, ventilation provides poorer protection against respiratory infections, and Callan and Garry (2002) pointed out that stocking density has a more dramatic effect on airborne pathogen density than ventilation. A two-fold increase in stocking rate requires nearly a 10-fold increase in ventilation to maintain the same airborne pathogen density, so ventilation cannot overcome grossly inadequate housing, management or hygiene with the barn.

Pathogenesis and transmission of infection

Since *M. bovis* colonizes the mucosal surfaces, frequently without causing clinical disease, and from there relocates to other organs, it can result in a wide range of severe clinical signs such as pneumonia, arthritis, mastitis and sometimes infections in the genital tract in adults and decubital abscesses (Kinde et al., 1993) . Calves often also frequently suffer from otitis media in addition to respiratory disease and arthritis. The upper respiratory tract and the mammary glands are the most important sites of persistence and shedding of the agent. Transmission from an infected cow to an uninfected cow is most often by udder-to-udder, via milking e and milkers' hands (Aebi et al., 2012). Transmission via respiratory secretions, aerosols and nose-to-nose-contact are also plausible routes of transmission (Maunsell et al., 2011). *M. bovis* has been isolated from air in barns containing diseased calves, and since calves can be experimentally infected by inhalation of *M. bovis* it is likely that transmission can occur airborne over short distances (Jasper et al., 1974; Nicholas et al., 2002). The incubation period ranges from two to six days and depends on the degree of mycoplasma circulation in the herd.

Farm to farm transmission

Apart from the well-known risk of moving cattle between farms (Aebi et al., 2015; Amram et al., 2013; Gonzalez et al., 1992), a recent Danish study based on four bulk-tank milk screening rounds of all dairy herds also found that proximity to test-positive dairy herds poses a marked risk of becoming test-positive in other dairy farms (unpublished data), and short-term clusters of infection were evident indicating that the infection moves fairly rapidly between susceptible farms when no control measures are in place to prevent it (Arede et al., 2016). In addition, participation in animal shows and markets leads to a risk of introduction of *M. bovis* to cattle farms, not only in the participating farm, but also in other contact farms in the trade network. Even though the 'diffuse transmission routes' associated the proximity risk have not been identified, theories certainly include long-distance airborne transmission and spread via other fomites including tools that people bring with them on farm at herd visits. Hence, it is recommended to carry as little as possible into farms and leave as much as possible behind on farm. Equipment that must be brought in should be disinfected before departure by wiping off, for instance with chlorhexidine solution.

Transmission between humans and cattle

To the best of my knowledge there is no evidence of human-to-cattle transmission, and only one study was identified in the literature suggesting that people can be infected with *M. bovis.* This was a case report about a severely ill patient in USA, who most likely got infected via contact to cow manure (Madoff et al., 1979). She was treated and recovered with tetracycline treatment. In other words, it cannot be entirely

ruled out that cross-species infection can occur, but it must be very rare. Hence, it is advisable that professionals do not to enter cattle farms, if they are suffering from respiratory disease and it is recommended to be extra careful with hygienic measures (i.e. wear gloves, change clothes, wash and disinfect boots, tools etc. used on farm) in outbreak farms, where the risk of carrying the pathogen to other farms is highest. Visitors should always change the outer layer of clothes and wash hands carefully after being in contact with animals in another farm, and boots should preferably be left on farm or must be cleaned properly and disinfected between farms, for instance by being placed in disinfecting fluid in a bucket while driving between farms.

Diagnostic methods

As stated by Ball and Nicholas (2010): 'Over recent years ELISA- and PCR-based methods have gradually replaced culture as the method of choice for detecting *M. bovis*, and the application of a novel real-time (RT)-PCR method …… makes a valuable new contribution in this context (Sachse et al., 2010). While sharing the advantages of high sensitivity and rapid sample throughput with traditional PCR methods, RT-PCR offers increased test specificity and does not require additional sample handling after the amplification stage, thus reducing the risk of cross-contamination'. Many research groups and companies worldwide are developing RT-PCR methods for improved and rapid detection of *M. bovis*, both for herd level and animal level diagnosis. However, the challenge remains to accurately detect persistent, infectious carriers for culling to improve the control measures without culling too many false-positive cattle. PCR (and culture) method are, however, still best for herds with mastitis problems, and in Denmark we have experienced quite some day-to-day variation in the PCR-results from infected farms.

ELISA-techniques for testing of antibodies in serum or milk samples is also increasingly popular due to the low price and rapid assessments, and they tests pose the currently best diagnostics for herd diagnosis in herds with arthritis and pneumonia cases, and for regular screening of *M. bovis*-free herds in connection with the animal trade or participation in shows, as well as for livestock monitoring in general. There are two main factors limiting the use of antibody detection ELISAs. First, antibody titres to *M. bovis* emerge only 10 to 14 days after the onset of disease, so that infection by the pathogen cannot be detected during the incubation period. Secondly, the sensitivity of this method is insufficient to identify shedders. Preferably a minimum of 15 random or samples from suspected infected animals per herd should be collected for herd diagnosis, more in large farms. In general, herds with more than 15% ELISA-positive samples are likely to have on-going transmission of *M. bovis* and in that case the test-negative animals from the same herd may well pose a risk of infection as well.

Control options

Stress-reducing farm management and actions that produce barriers to wide-spread transmission of high concentrations of bacteria - particular via milk and aerosols - are important. This means that keeping good barn hygiene and minimizing direct contact between many animals are essential. Hence, housing of large groups, movement of animals between groups and lack of separation between groups are significant risks. Also, stocking density and the way the animals are handled by the caretakers is very important for the level of stress affecting the cattle in the farm.

Pasteurisation of milk fed to calves or feeding good milk replacers to calves is highly recommended in farms with *M. bovis*-associated mastitis and large farms where transmission is most difficult to control. Separation of sick from healthy animals is essential as they are excreting high concentrations of bacteria through the airways. Hence, sick-pens (not sick-pens together with the calving areas) or separate sick-calf hutches should always be used for sick animals.

One study found 0.5% sodium hypochlorite or 2% chlorhexidine efficacious in eliminating *Mycoplasma spp*. from contaminated bedding sand (Justice-Allen et al., 2010), and this disinfectant can be used for other equipment as well.

Elimination of *M. bovis* from an infected herd is possible by following a herd-specific plan including both management and hygiene measures together with testing procedures to try to identify carrier animals (Bicknell et al., 1983).

The recommendations by Jørgen Katholm (2014) on how to control *M. bovis* in test-positive dairy herds are as follows: (http://dna-diagnostic.com/media/33656/eradication-and-handling-guidelines-for-mycoplasma-bovis.pdf)

- Optimize hygiene on farm.
- Optimize hygiene at milking. Milk with gloves and do post milk teat dipping with iodine.
- Optimize feeding and all other stress factors on the farm with focus on movements.
- Never place sick cows in the fresh cow pen.
- Stop feeding fresh cow milk to calves.
- Only feed calves from dam with colostrum 2-4 days.
- Pasteurize all other milk from cows to calves or use milk powder.
- Test cows treated the last 1-2 months for clinical mastitis with composite milk samples for *M. bovis* by PCR and segregate positive cows.
- Test all new mastitis cases the next 1-2 month for *M. bovis* with PCR and segregate positive cows.
- Stop milking all positive cows with clinical mastitis and positive reaction for *M. bovis*, move these cows to fattening area away from the milking cows or cull.
- Test bulk tank milk samples with PCR every 1-2 week until negative results.

If handled well, outbreaks rarely last beyond 3 months. And losses can be limited, if all the above recommendations are followed. Herd immunity will eventually help clear the infection from the herds.

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